

Altered Vitamin B₁₂ Metabolism in Fibroblasts from a Patient with Megaloblastic Anemia and Homocystinuria Due to a New Defect in Methionine Biosynthesis

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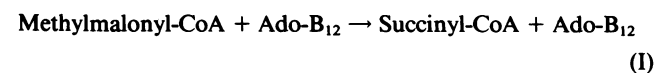
Abstract. Cultured fibroblasts from a recently described patient with homocystinuria and megaloblastic anemia of infancy without methylmalonic aciduria were previously shown to have normal cobalamin uptake and a specific decrease in the proportion of intracellular methylcobalamin. As in control cells but unlike in those from patients with combined homocystinuria and methylmalonic aciduria (cobalamin C and cobalamin D), accumulated ⁵⁷Co-labeled cobalamin was bound in appropriate amounts and proportion to intracellular binders which are known to be the two vitamin B₁₂-dependent enzymes, methionine synthetase and methylmalonyl-CoA mutase. Despite the association of a normal quantity of intracellular cobalamin with methionine synthetase, the proportion of intracellular cobalamin which was methyl-B₁₂ was below normal and in the range observed in cobalamin C and D cells. This methyl-B₁₂ was decreased by exposure of fibroblasts in culture to nitrous oxide as was observed with control cells. Exposure of control fibroblasts during culture, but not of fibroblasts from this patient, to nitrous oxide significantly reduced

the holoenzyme activity of methionine synthetase assayed in cell extracts. In addition, although methionine synthetase activity in cell extracts of control and cells from the patient were similar in the presence of standard assay concentrations of thiols, at low thiol concentrations, methionine synthetase activity in extracts of cells from the patient was much lower than in control extracts. Mixing of control patient extracts corrected this decreased activity in excess of that explained by addition of the individual activities added. The defect of this patient appears to be in a reducing system required for methionine synthesis.

Introduction

Inherited disorders affecting vitamin B₁₂ include those affecting transport and those affecting intracellular metabolism. Transport defects include inherited abnormalities of gastrointestinal absorption due to either abnormal transport protein (intrinsic factor) or unexplained defects in translocation of vitamin from lumen to portal plasma. Defective transfer of vitamin B₁₂ after absorption is caused by absent or defective transcobalamin II which facilitates entry of circulating vitamin B₁₂ into cells.

Coenzyme forms of vitamin B₁₂ (cobalamin) in mammalian cells have been shown to mediate enzyme reactions generating succinyl-coenzyme A from methylmalonyl-coenzyme A (reaction I), methionine from homocysteine (reaction II), and interconverting alpha and beta leucine (reaction III):



Inherited defects of intracellular metabolism that have been described include those affecting only synthesis of

This is publication No. 84003 of the McGill University-Montreal Children's Hospital Research Institute. Dr. Cooper is a Career Investigator of the Medical Research Council of Canada. Dr. Rosenblatt is a Principal Investigator in the Medical Research Council of Canada Genetics Group. Address reprint requests to Dr. Rosenblatt, MRC Genetics Group, 2300 Tupper Street, A709, Montreal, Quebec, Canada, H3H 1P3.

Received for publication 24 January 1984 and in revised form 16 August 1984.

J. Clin. Invest.

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0021-9738/84/12/2149/08 \$1.00

Volume 74, December 1984, 2149-2156

5' deoxyadenosyl cobalamin (Ado-B₁₂)¹ which produce methylmalonic aciduria without homocystinuria (cobalamin A and B disease) and those which affect the synthesis of both Ado-B₁₂ and methylcobalamin (CH₃-B₁₂) which produce homocystinuria and homocystinemia without increased plasma methionine, and also produce methylmalonic aciduria with or without megaloblastic anemia (cobalamin C and D diseases).

We have recently described a patient who presented with megaloblastic anemia and homocystinuria but no methylmalonic aciduria (1). Fibroblasts from this patient required extracellular methionine for growth, unlike normal fibroblasts which could grow on homocysteine, and had impaired incorporation of labeled methyl from 5-CH₃-H₄PteGlu into protein, indicating defective synthesis of methionine. Unlike cells from patients with inherited defects described previously, total cobalamin incorporation was normal in these cells. Intracellular Ado-B₁₂ was normal but CH₃-B₁₂ was decreased. Despite evidence of defective synthesis of methionine within intact cells, methionine synthetase (5-methyltetrahydropteroyl-L-glutamate:L-homocysteine S-methyltransferase; E.C. 2.1.1.13) activity assayed in cell extracts was normal. The present studies suggest that this patient has an abnormality of methionine biosynthesis *in vivo* which is overcome in the standard assay procedure for methionine synthetase. This defect is probably due to failure of reduction of B₁₂ bound to methionine synthetase.

Methods

Materials. Tissue culture medium was purchased from Flow Laboratories (Rockville, MD). Folate- and methionine-deficient medium was purchased from Grand Island Biological Co. (Grand Island, NY). [¹⁴C]methyltetrahydrofolate (CH₃H₄PteGlu) and [⁵⁷Co]cyanocobalamin (CN-B₁₂) were purchased from Amersham Corp. (Oakville, Ont.). For use in the methionine synthetase assay, [¹⁴C]CH₃H₄PteGlu was purified on a column (0.9 × 55 cm) of Sephadex G25 (Pharmacia, Uppsala, Sweden) according to the method of Mellman et al. (2). CN-B₁₂, hydroxocobalamin (OH-B₁₂), CH₃-B₁₂, and Ado-B₁₂ were purchased from Sigma Chemical Co. (St. Louis, MO). [⁵⁷Co]OH-B₁₂ was prepared from [⁵⁷Co]CN-B₁₂ (3) and verified as OH-B₁₂ by its mobility on high pressure liquid chromatography.

Cell cultures. Fibroblasts were obtained from a patient with megaloblastic anemia and homocystinuria by Dr. J. C. Haworth, Dept. of Pediatrics, University of Manitoba. This patient has been the subject of a previous report (1). Control fibroblasts were obtained from the Repository for Mutant Human Cells, Montreal Children's Hospital. All lines were tested for mycoplasma contamination (4). Cells were grown in glass roller bottles (Bellco Glass Inc., Vineland, NJ) of 690 cm² surface area, in plastic roller bottles (Corning Glass Works,

Corning, NY), or in 32-oz glass bottles. The standard medium consisted of Eagle's minimum essential medium which contains PteGlu (2.3 μM) plus nonessential amino acids (5, 6). Routinely, the medium contained 10% fetal calf serum, dialyzed where indicated against a 10-fold excess of 0.9% NaCl with two changes. For cobalamin uptake experiments using [⁵⁷Co]CN-B₁₂, medium contained 10% human serum which had been preincubated with labeled CN-B₁₂ as described by Mellman et al. (2). All cultures containing cobalamin derivatives were both incubated and harvested in the dark. Cells were released from the culture vessels by exposure to 0.25% trypsin for 10 min at 37°C and resuspended in phosphate-buffered saline (pH 7.4), and an aliquot was removed for enumeration in a Coulter counter (Coulter Electronics, Hialeah, FL). The cell pellet was washed twice in phosphate-buffered saline and collected by centrifugation.

Assay of methionine synthetase activity. Confluent fibroblasts, cultured as described above were resuspended at a final concentration of 10⁸ cells/ml in 0.1 M potassium phosphate (pH 7.4). The cell suspension was sonicated on ice using four 20-s blasts with intervals of 20 s between blasts. The sonicates were centrifuged for 10 min × 48,000 g and the supernatant used. This supernatant has been designated as "cell extract" or "extract." Assay of the methionine synthetase activity was performed essentially according to the method of Mellman et al. (2). In a total volume of 0.2 ml, the reaction mixture consisted of 100 mM potassium phosphate (pH 7.4), 250 μM S-adenosylmethionine (purified where indicated by elution from a Dowex-1-bicarbonate column according to the procedure of Shapiro and Ehninger [7]), 500 μM DL-homocysteine (prepared fresh daily from the thiolactone), 50 μM CH₃-B₁₂, 390 μM [¹⁴C]CH₃H₄PteGlu (1.4 dpm/pmol), and 0.4–3 mg cell protein. The standard assay contained 150 mM beta mercaptoethanol (BME). This was substituted with the indicated concentrations of dithiothreitol (DTT) for some experiments. The reaction was carried out in sealed Vacutainer tubes (Becton-Dickinson, Mississauga, Ontario, Canada) which had been flushed with nitrogen for 5 s. Incubation in the dark in a heated aluminum block (Buchler Instruments, Inc., Fort Lee, NJ) was for 90 min at 37°C. The reaction was terminated by the addition of 0.8 ml ice-cold distilled water. The samples were applied to a syringe column with a bed volume of 1.5 ml Bio-Rad AG 1 × 8 resin, 200–400 mesh, chloride form (Bio-Rad Laboratories, Richmond, CA) and the columns were washed with an additional 1 ml water. The entire effluent of 2 ml was collected directly into a scintillation vial and counted in 16 ml New England Nuclear Formula 963 counting solution (New England Nuclear, Boston, MA) in a Packard Liquid scintillation counter (Packard Instruments Co., Downers Grove, IL).

Intact cell assays. The determination of the incorporation of [¹⁴C]CH₃H₄PteGlu and [¹⁴C]propionate into acid-precipitable material were performed according to the methods of Mellman et al. (2).

Gel filtrations and polyacrylamide gel electrophoresis. Both these procedures were performed according to the method of Mellman et al. (2).

Effect of nitrous oxide. Roller bottles containing control and the patient's fibroblasts were incubated for 3 or 4 d in the dark in the standard culture medium to which was added 0.75 μM OH-B₁₂. 16 h before harvesting the bottles were gassed for 2 min with a mixture (flow rate of 5 liter/min each) of 50% nitrous oxide:50% oxygen (Union Carbide Canada Ltd., Toronto, Ontario). Cells were harvested in the dark for the methionine synthetase assay as described above.

Fractionation of intracellular cobalamins. Washed pellets of cells grown in 25 pg/ml of labeled cobalamin were extracted (8) in complete

1. *Abbreviations used in this paper:* Ado-B₁₂, 5' deoxyadenosylcobalamin; AdoMet, S-adenosylmethionine; BME, beta mercaptoethanol; CH₃-B₁₂, methylcobalamin; CH₃H₄PteGlu, methyltetrahydrofolate; CN-B₁₂, cyanocobalamin; DTT, dithiothreitol; H₄PteGlu, tetrahydrofolate; OH-B₁₂, hydroxocobalamin.

darkness in 10 ml of absolute ethanol at 85°C for 20 min and centrifuged, and 8 ml of supernatant ethanol was evaporated to dryness under a stream of nitrogen at room temperature. The dry residue was dissolved in 1 ml of water and mixed with 10 ml of ether followed by separation of ether by centrifugation. 8 ml of separated ether was discarded, and the remainder evaporated under a stream of nitrogen gas at room temperature. The remaining 1 ml aqueous sample was applied to a column of SP Sephadex C-25 (35 × 1.6 cm) (9). 30 3.5-ml fractions were eluted with water followed by 30 fractions eluted with 0.05 M sodium acetate, pH 5, and 40 fractions eluted with 0.1 N NaOH to strip the column. When standards of CN-B₁₂, OH-B₁₂, CH₃-B₁₂, and Ado-B₁₂ were so treated and fractionated, they were eluted in fractions 10–12, 14–18, 45–55, and 69–80, respectively. Following completion of most of the fractionations described here, some were reanalysed on high pressure liquid chromatography using a Merck column Lichrosorb RP-8 (E. Merck, Darmstadt, West Germany), 10 μm eluted with a gradient of phosphate at pH 3 and triethylammonium phosphate as described by Jacobsen et al. (10). For high

pressure liquid chromatography fractionation, extraction was identical to that above except that extraction with ether was omitted.

Results

Accumulation and distribution of labeled cobalamin by mutant cells. As reported previously, accumulation of label from [⁵⁷Co]CN-B₁₂ in culture medium over 4 d was equal in cells of the patient and normal cells, whereas the previously described defective accumulation of cobalamin by fibroblasts from patients with cobalamin C and D disease was observed. The majority of label associated with normal and patient's fibroblasts filtered through Sephadex G-150 as bound cobalamin, filtering just within the included volume of the gel (Fig. 1). This bound cobalamin separated into two fractions during electrophoresis on polyacrylamide gel, representing cobalamin bound to methylmalonyl-CoA mutase (slower migrating fraction) and methi-

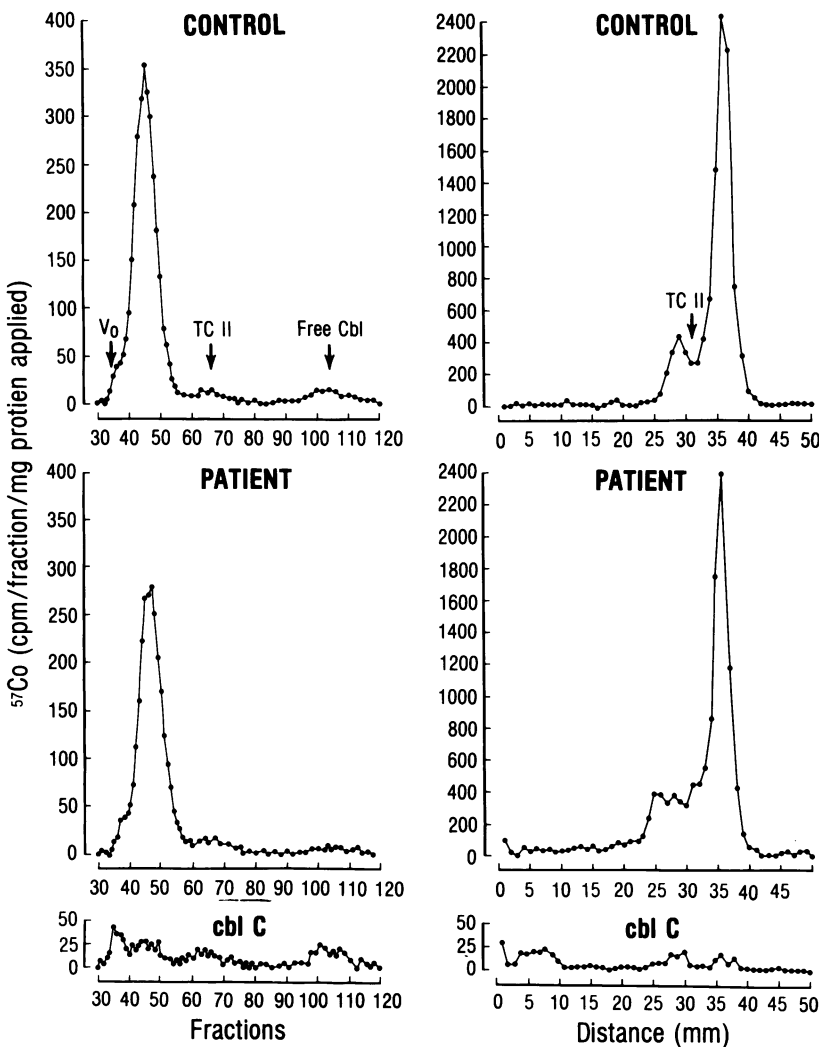


Figure 1. [⁵⁷Co]cobalamin binding of fibroblasts extract from a control, our patient, and a cobalamin (cbl) C patient after Sephadex G-150 gel filtration (left) and polyacrylamide-gel electrophoresis (right). After Sephadex G-150 gel filtration, both methionine synthetase and methylmalonyl-CoA mutase activities have been shown (2) to elute in a single fraction, seen in this experiment to have a peak at fraction 45. After polyacrylamide-gel electrophoresis, methionine synthetase and methylmalonyl-CoA mutase have been shown (2) to elute as separate fractions, seen in this experiment to have peaks at fractions 37 and 28, respectively.

onine synthetase. The proportion of intracellular label associated with the methionine synthetase (fractions 33–38; Fig. 1, right) was similar in both normal cells and the patient's cells, and much less in cells from a cobalamin C patient. It was thus apparent that unlike cobalamin C cells, cells from the proband accumulated normal quantities of cobalamin and bound a normal proportion of this to methionine synthetase enzyme.

Labeled cobalamins extracted from fibroblasts incubated in [^{57}Co]CN-B $_{12}$ were eluted from SP-Sephadex C-25 in five distinct and sequential fractions. Studies with standards and with photolability established that the second, third, fourth, and fifth fraction eluted contained CN-B $_{12}$, CH $_3$ -B $_{12}$, Ado-B $_{12}$, and OH-B $_{12}$, respectively. Aquocobalamin (H $_2$ O-B $_{12}$) was found to elute in the first fraction as did some of vitamin B $_{12}$ (Co $^{+2}$) and B $_{12}$ (Co $^{+1}$) applied to the column, probably because of spontaneous oxidation during chromatography. Sulfitecobalamin (SO $_3$ -B $_{12}$) has been reported to elute in this fraction as well (11). The chemical nature of the intracellular cobalamins eluting in the first fraction could thus not be reliably identified, but this first fraction from fibroblasts was photostable and thus probably was not sulfitecobalamin.

Extracts were prepared from six different cell lines of control fibroblasts and the cobalamin profile determined. One of these lines was grown and analyzed on six separate occasions. The distribution of individual cobalamins in normal cells (N), in repeated determinations in patient's cells (P), and in two

different cell lines of cobalamin C and D mutants are plotted in Fig. 2. In this figure, single solid symbols represent individual determinations of cobalamin distribution. Values obtained in repeated determinations using a single cell line are indicated by vertical bars representing the standard deviation of these repeated analyses. It is apparent that cells from the patient (P) and cobalamin C and D mutants contained more CN-B $_{12}$ and less CH $_3$ -B $_{12}$ than did normal cells, and that cobalamin C and D cells contained less Ado-B $_{12}$ than did normal or patient's cells ($P < 0.05$, Student t test).

Effect of modification of methionine synthetase assay on activity measured in the patient's cells. Screening tests for cobalamin-dependent enzymes had revealed defective incorporation of [^{14}C]CH $_3$ H $_4$ PteGlu into the patient's cells with normal incorporation of [^{14}C]propionate. These studies had indicated defective methionine synthesis from CH $_3$ -H $_4$ PteGlu and homocysteine, but normal succinate synthesis from methylmalonyl-CoA. These findings were consistent with the defective CH $_3$ -B $_{12}$ and normal Ado-B $_{12}$ observed in the patient's cells because of the requirement of these reactions for these cobalamins, respectively. Activity of methionine synthetase assayed *in vitro* in cell extracts was normal when the assay utilized conditions described for optimal activity (150 mM BME) (1). This is consistent with the normal cobalamin content of the methionine synthetase enzyme shown in Fig. 1.

When concentrations of most reactants were varied under

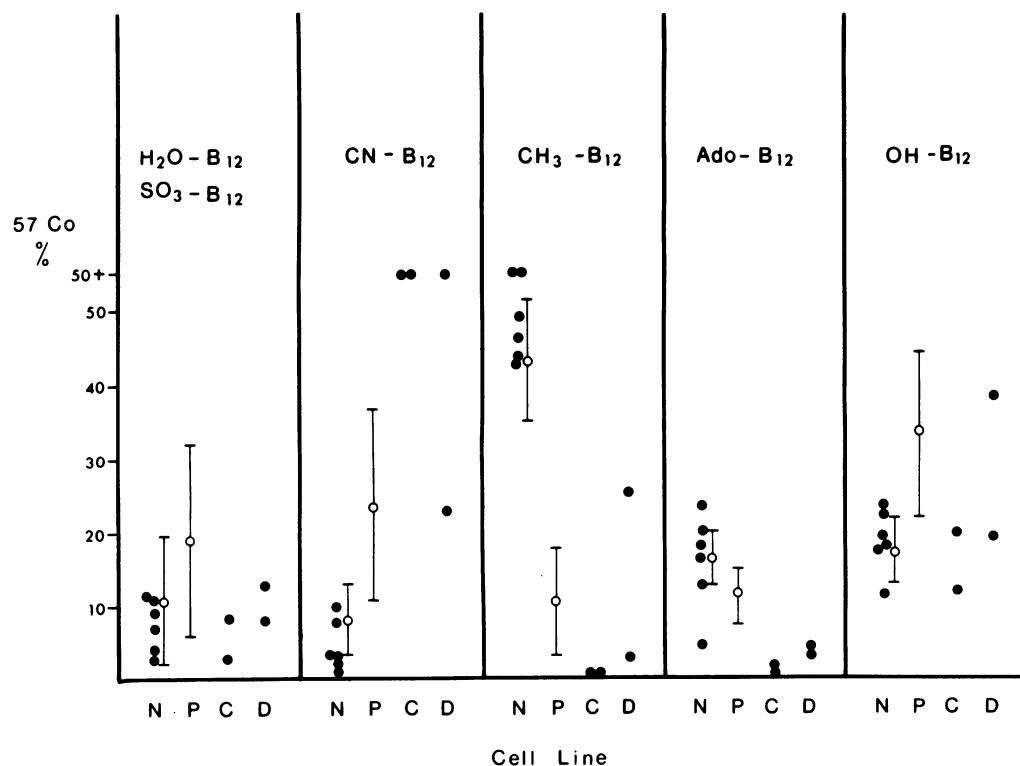


Figure 2. Distribution of cobalamin forms in extracts of fibroblasts from normal cell lines (N), patient's fibroblasts (P), and two different cell lines of cobalamin C and D disease (C and D, respectively). Solid symbols represent single determinations of distribution of ^{57}Co -activity in extracts of cells grown in 25 $\mu\text{g/ml}$ (18 μM) of ^{57}Co -labeled CN-B $_{12}$ for 3 d. Open symbols represent mean values for cell lines examined repeatedly, and vertical bars represent 1 SD. One line of normal fibroblasts was examined six times, and the patient's fibroblasts were examined nine times.

standard reducing conditions (150 mM BME), no difference was observed between the effect of such modifications on the enzyme activity in normal and the patient's cells. Reactants with similar sensitivity in affecting enzyme activity in normal and the patient's cells included $\text{CH}_3\text{-H}_4\text{PteGlu}$, S-adenosyl-methionine (AdoMet), and $\text{CH}_3\text{-B}_{12}$. When the concentration of reducing agent in the reaction was altered, however, enzyme activity in the patient's extract became less than that of control extract at similar levels of reducing agent (Fig. 3). The greatest difference between methionine synthetase activity in extracts of control and the patient's cells was observed at low concentrations of DTT. In addition to this greater requirement for DTT of methionine synthetase activity in patient's extracts than in control extracts, patient's extracts almost always generated more methionine synthetase activity in BME than in DTT, whereas the reverse was consistently observed in extracts of control cells. The ratio of methionine synthetase activity in 150 mM of BME to that in 25 mM DTT was 0.85 ± 0.13 and 0.86 ± 0.10 ($x \pm \text{SD}$, $n = 3$) in two control extracts and 1.45 ± 0.26 ($n = 4$) in patient's extracts. At low concentration of BME or DTT, however, the methionine synthetase activity in extracts of control cells was always greater than that of the patient's cells. Methionine synthetase activity was thus consistently different in the patient's extracts from that in control extracts in that more activity was lost when reducing conditions were less stringent and in being more responsive to BME than to DTT as a reducing agent.

Effect of mixing control and patient extracts on methionine synthetase activity. To determine if a factor other than methi-

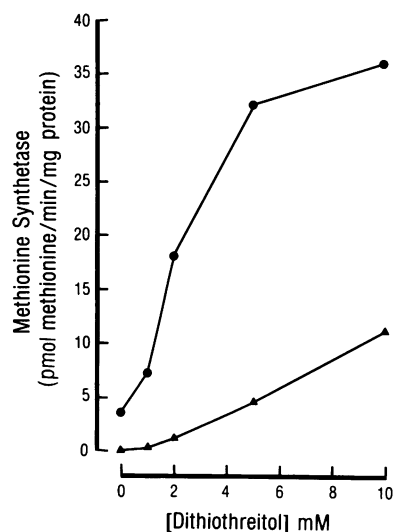


Figure 3. Effect of varying concentrations of DTT on the activity of methionine synthetase in extracts from the patient and a control. Methionine synthetase activity was determined without BME, but with different concentrations of DTT in the reaction mixture. Values are the mean of duplicate determinations from one representative experiments. —●—, control; —▲—, patient.

onine synthetase might be defective in extracts of patient's cells, methionine synthetase was analysed at 25 mM DTT (a concentration at which activity is maximal in normal but not patient's cells) in mixtures of normal and patient cells (Table I). Under these conditions, small quantities of extract from control cells were found to correct the methionine synthetase activity of mutant cells in excess of the methionine synthetase activity added from the normal extract. Mixing control and patient extract at 150 mM BME was only additive, and cobalamin C and D extract also corrected the defects in similar experiments at 10 mM DTT (data not shown). This indicates that the defect in the extracts of the patient's cells was not restricted to the methionine synthetase enzyme, but appeared due to the lack of an additional factor which corrected the activity of methionine synthetase in extracts of patient's cells.

Because it appeared that the intracellular defect related to reduction of cobalamin in patient cells, cells were incubated with different concentrations of DTT and ^{14}C -labeled $\text{CH}_3\text{-H}_4\text{PteGlu}$ and unlabeled OH-B_{12} to determine if this would affect the incorporation of labeled methyl into protein. Growth was inhibited at DTT concentrations in excess of 1 mM, but neither this concentration nor 10 or 20 mM DTT altered the incorporation of labeled methyl groups into normal or mutant cells, suggesting no effect on methionine synthetase within the cells. Growth of cells in the dark in $1.5 \mu\text{M}$ $\text{CH}_3\text{-B}_{12}$ in addition to 1 mM DTT did not improve incorporation of labeled methyl by cells from the patient despite chromatographic evidence that >90% of the extracellular cobalamin was $\text{CH}_3\text{-B}_{12}$ at the end of the incubation.

Effect of nitrous oxide on methionine synthetase activity of cultured cells. The above studies indicated that whereas methionine synthetase activity in standard concentrations of thiols was normal in extracts of patient's cells, failure of the cells to synthesize adequate methionine for growth and their

Table I. Effect of Mixing Extract from Patient with Control Extract on Methionine Synthetase Activity

Cell line	Protein added mg	Methionine formed pmol/min
Control	0.6	31
	1.3	72
Patient	0.7	24
	1.5	35
Control + Patient	0.3+1.5	77 (51)
	0.6+1.5	102 (66)

Methionine synthetase activity was determined using 25 mM DTT as a reducing agent. At this concentration of reducing agent, control activity but not patient activity is maximal. Incubation was for 90 min at 37°C . Values are the average of duplicate determinations. The expected values for additive mixing are shown in brackets.

reduced incorporation of [^{14}C]methyl from 5- $\text{CH}_3\text{-H}_4\text{PteGlu}$ compared with normal cells suggested that methionine synthetase activity was not normal within the living cell. Nitrous oxide has been shown to irreversibly inhibit methionine synthetase within cells probably by oxidation of $\text{B}_{12\text{s}}$ (Co^{+1}) to inactive oxidation products. Nitrous oxide does not attack $\text{CH}_3\text{-B}_{12}$, but appears to require transfer of the CH_3 -groups as the enzyme turns over to leave $\text{B}_{12\text{s}}$ (Co^{+1}) which can be attacked by the gas. If methionine synthetase was less active in patient's cells than in normal cells, less inhibition of methionine synthetase activity by nitrous oxide should be observed.

Fibroblast monolayers were incubated in an excess (0.75 μM) of OH-B_{12} for 3 d, and at 18 h before the end of the incubation, culture bottles were gassed with 50% of nitrous oxide in oxygen. Holoenzyme activity of methionine synthetase activity (standard conditions) was reduced to <10% of control activity in extracts of control cells, whereas >75% of holoenzyme activity was retained by extracts of patient's cells (Fig. 4). Apoenzyme activity (total holoenzyme) was not significantly affected by nitrous oxide exposure, indicating no effect of the gas on the enzyme itself.

When fibroblasts were incubated for 3 d in labeled cobalamin with a source of transcobalamin II (human serum) and

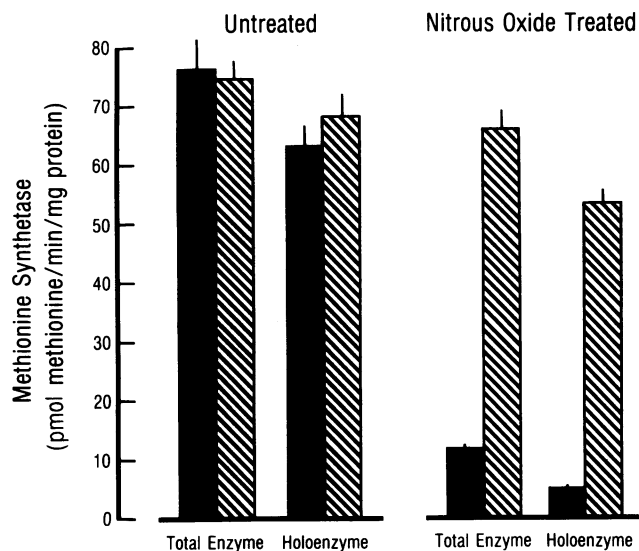


Figure 4. Effect of nitrous oxide on methionine synthetase activity of cultured fibroblasts preincubated in OH-B_{12} . Fibroblasts from a control and from the patient were incubated in medium containing 0.75 μM OH-B_{12} for 4 d. 16 h prior to harvesting, replicate roller bottles were gassed with a mixture of nitrous oxide and oxygen (1:1). The cells were harvested and assayed for methionine synthetase activity in the presence (total enzyme) or the absence (holoenzyme) of $\text{CH}_3\text{-B}_{12}$ in the reaction mixture. Incubation was for 90 min at 37°C in the standard assay mixture containing BME. Values are the average of triplicate determinations \pm SD from one representative experiment. ■, control; ▨, patient.

exposed to nitrous oxide for 18 h, ^{57}Co -labeled $\text{CH}_3\text{-B}_{12}$ was reduced to 4–8% of total intracellular cobalamin in both control and patient's cells. In addition, directly exposing cell extracts to nitrous oxide for 1 min prior to assay resulted in a similar decrease in methionine synthetase specific activity by 40% in control and by 48% in patient's cells. This demonstrates that the proportion of methionine synthetase enzyme which was active corresponded to the proportion of $\text{CH}_3\text{-B}_{12}$ bound to it, and that in patient's cells, although the total proportion of the enzyme binding vitamin B_{12} was similar to that in normal cells, only a minority of this bound cobalamin was $\text{CH}_3\text{-B}_{12}$, turned over intracellularly, and could be inhibited with nitrous oxide. The remainder of the enzyme-bound cobalamin could be activated in extracts in the presence of thiols, probably by conversion to $\text{B}_{12\text{r}}$ (Co^{+2}). At concentrations of thiols used in the assay, solutions of OH-B_{12} could be shown to be converted to $\text{B}_{12\text{r}}$ (Co^{+2}) within 10 min of adding DTT or BME. This strongly suggests that the majority of cobalamin bound to methionine synthetase in patient's cells was OH-B_{12} (Co^{+3}) or $\text{OH}_2\text{-B}_{12}$ (Co^{+3}) since these forms would not be affected by nitrous oxide but would be activated in vitro by DTT or BME in the standard assay.

Discussion

Fibroblasts from this patient with homocystinuria and megaloblastic anemia in infancy were shown in a previous report (1) to incorporate labeled cobalamin in a normal fashion but to contain a smaller proportion of $\text{CH}_3\text{-B}_{12}$ than did normal cells. They were found to utilize CH_3 from $\text{CH}_3\text{-H}_4\text{PteGlu}$ less effectively than did normal cells. They differed from previously reported inherited defects of cobalamin metabolism (cobalamin C and D disease) in containing normal quantities of Ado-B_{12} , in utilizing labeled propionate in a normal manner, and in having normal activity of methionine synthetase in cell extracts under standard assay conditions. Thus the defect in this child involves selectively the metabolism of $\text{CH}_3\text{-B}_{12}$ but not Ado-B_{12} . This is supported by the failure of fibroblasts from the patient to grow in tissue culture medium in which homocysteine replaced methionine, even in the presence of excess B_{12} . Normal levels of methylmalonic acid in the urine indicate that the same defect affected the whole patient. The defect in the child appeared less complete than in cultured fibroblasts, since the homocystinuria and megaloblastic anemia responded completely to large doses of OH-B_{12} .

In this study we report that the intracellular cobalamin of fibroblasts obtained from this patient is distributed between methionine synthetase and methylmalonyl-CoA mutase as in normal cells and unlike in cobalamin C cells. About two-thirds of intracellular cobalamin in normal fibroblasts is associated with methionine synthetase and approximately the same proportion of intracellular cobalamin is present as $\text{CH}_3\text{-B}_{12}$, which is generated from vitamin B_{12} bound to this enzyme. The disparity between vitamin B_{12} associated with methionine synthetase and the smaller concentration of $\text{CH}_3\text{-B}_{12}$ in the

patient's than in the normal cells implies that much of the B₁₂ associated with methionine synthetase in the patient's cells is not CH₃-B₁₂. The lability of reduced cobalamins prevents the direct measurement of the form of vitamin B₁₂ associated with methionine synthetase. The above suggests that whereas methionine synthetase activity in cell extracts from the patient appeared to be normal under standard assay conditions, it may not be normal within the patient's cells.

Two abnormalities of methionine synthetase activity were observed in patient's cells: (a) a greater dependence of methionine synthesis in extracts of patient's cells to the concentration of thiols in the assay mixture with greater sensitivity to BME than to DTT; (b) resistance to the methionine synthetase activity within intact cells to irreversible inactivation by nitrous oxide. The former (a) suggests that much of the methionine synthetase enzyme in extracts of the patient's cells is either more labile to oxidation or is deficient in a reduction step required for methionine synthesis. The latter (b) suggests that much of the methionine synthetase enzyme is not active within the patient's cells, since inactivation by nitrous oxide follows methylation of homocysteine by methylcobalamin leaving residual B_{12s}(Co⁺¹) which is susceptible to oxidation by nitrous oxide (12). The normal sensitivity of the patient's methionine synthetase to inactivation by nitrous oxide in vitro and the decrease of intracellular methylcobalamin during incubation in nitrous oxide excludes an unusual enzyme configuration preventing access of the gas to the cobalamin. The correction of defective methionine synthesis in extracts of patient's cells by the addition of small quantities of normal extract suggests that the defect involves a step other than methionine synthetase, which might be related to the reduction of components during the enzyme reaction.

Methionine synthetase in *Escherichia coli* has been exten-

sively studied and appears to transfer a methyl group from 5-CH₃H₄PteGlu to B_{12s}(Co⁺¹) to form CH₃-B₁₂ with subsequent methylation of homocysteine (13). The enzyme appears to bind B_{12r}(Co⁺²) and to require AdoMet as a methyl donor to methylate B_{12r}(Co⁺²) to form methylcobalamin; then, 5-CH₃H₄PteGlu can function as a methyl donor until the resulting B_{12s}(Co⁺¹) oxidizes to B_{12r}(Co⁺²). The greater the reducing activity of the enzyme reaction, the smaller is the requirement for AdoMet and the more cycles of methylation of B_{12s}(Co⁺¹) by folate are observed before remethylation by AdoMet is required. Studies with mammalian enzymes have been consistent with a similar mechanism, but have been less extensive (13). Fujii and Hunnekins (14) reported that two flavoproteins might function as a coupled reducing system in methionine synthesis in *E. coli*. These materials replaced most requirements for exogenous reducing substances during in vitro reaction, but did not enhance synthetase activity above that generated by flavin adenine dinucleotide or thiols. If such a reducing system is required in mammalian cells, a component of it could be missing in the child studied (Fig. 3).

The role of reduction is unclear, but might function to maintain cobalamin on the enzyme in the B_{12r}(Co⁺²) state. We suggest that most of the cobalamin on the methionine synthetase enzyme in the patient's cells is B_{12a}(Co⁺³) which would be unaffected by nitrous oxide, but activated in vitro by thiols. The irreversible inactivation of enzyme-associated cobalamin by nitrous oxide probably involves oxidation to cobalamin analogues. Only the minority of enzyme-associated cobalamin competent to form CH₃-B₁₂ in the patient's cells would be susceptible to such inactivation during methionine synthesis. In control cells, all or most enzyme-associated cobalamin probably contributes to methionine synthesis and is susceptible to inactivation by nitrous oxide.

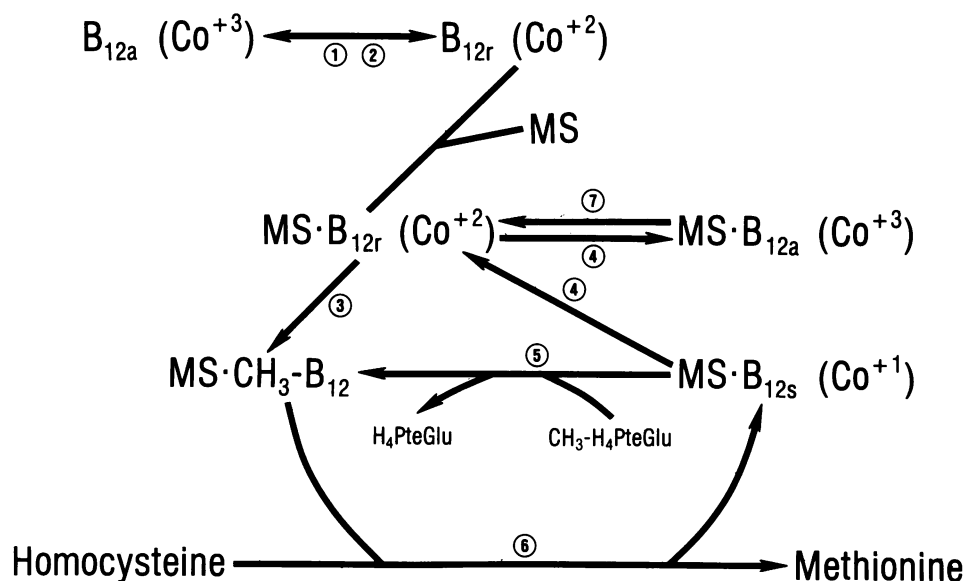


Figure 5. Suggested scheme of cobalamin involvement in methionine biosynthesis: 1 and 2, cobalamin reductase(s) postulated to be defective in cobalamin C and D mutants; 3, MS·B_{12r}(Co⁺²) + S-adenosylmethionine (AdoMet) → MS·CH₃-B₁₂ + 5' deoxyadenosylcobalamin (AdoHcy); 4, spontaneous oxidation of enzyme-bound B₁₂ to either Co⁺³ or Co⁺¹; 5, formation of MS·CH₃-B₁₂ from MS·B_{12s}(Co⁺¹) and CH₃-H₄PteGlu; 6, synthesis of methionine from MS·CH₃-B₁₂ and homocysteine; 7, putative B_{12a}(Co⁺³) reductase of enzyme-bound B_{12a} postulated to be deficient in our patient. MS, methionine synthetase enzyme.

These studies suggest that in this patient there is an abnormality of methionine synthesis which can be demonstrated in cell extracts when these are assayed under conditions of limited reduction. The abnormality induces intracellular deficiency of methylcobalamin secondary to the defective enzyme activity. It is suggested that the defect is in a reducing system normally responsible for maintaining enzyme-bound cobalamin as $B_{12}(Co^{+2})$. The defective molecule was excluded from Sephadex G-25 and thus probably is not unbound flavin or dinucleotide.

Cobalamin C and D cells, which have been assumed to be deficient in a cobalamin reductase activity (3), are defective in accumulating cobalamin from the surrounding medium in culture. Cells from the patient described here were not defective in accumulating cobalamin. If the cells of this patient were defective in a cobalamin reductase which reduces $B_{12a}(Co^{+3})$ bound to methionine synthetase, but the putative reductase system deficient in cobalamin C and D cells were intact, one might explain this with the following hypothesis (Fig. 5): It is probable that binding of cobalamin entering the cytoplasm to methionine synthetase is required to permit the accumulation of cobalamin by cells. If oxidized cobalamin entering the cytoplasm from lysosomes required reduction to bind to methionine synthetase, but once bound, could be oxidized without displacement from the enzyme, then cells defective in reductases acting on free cobalamin would not accumulate cobalamin effectively (cobalamins C and D); those with reductase defects affecting only the bound cobalamins (our patient) would accumulate B_{12} normally, but would accumulate oxidized and thus unusable cobalamins during the course of intracellular metabolism. If so, intracellular cobalamin accumulation would require reduction of $OH-B_{12}(Co^{+3})$ entering the lysosomes, and accumulation in our patient's cells would be expected to be normal. Once on the methionine synthetase, oxidation of reduced $B_{12}(Co^{+2})$ to $OH-B_{12}(Co^{+3})$ is not associated with the loss of cobalamin from bacterial enzyme (13). In our patient, such reoxidized cobalamin would be available for methionine synthesis in cell extracts if reduced by thiols, but would not function within intact cells because of a putative deficiency of a reducing activity associated with methionine synthetase.

Acknowledgments

We want to thank Dr. Robert T. Taylor for continued advice and suggestions. We also wish to thank Dr. J. C. Haworth for providing access to fibroblasts from this patient and Dr. L. E. Rosenberg for providing fibroblasts from cobalamin C and cobalamin D patients. We are grateful to Drs. R. E. MacKenzie, V. M. Whitehead, M. Parniak, and J. Jolivet of the Montreal Folate Club for advice and encouragement. We thank Lynne Prevost for preparation of the manuscript.

This research was funded by a Medical Research Council of Canada Group Grant in Genetics to Dr. Rosenblatt and operating grant MT802 to Dr. Cooper.

References

- Schuh, S., D. S. Rosenblatt, B. A. Cooper, M.-L. Schroeder, A. J. Bishop, L. E. Seargeant, and J. C. Haworth. 1984. Homocystinuria and megaloblastic anemia responsive to vitamin B_{12} therapy; an inborn error of metabolism due to a defect in cobalamin metabolism. *N. Engl. J. Med.* 310:686-690.
- Mellman, I., H. F. Willard, and L. E. Rosenberg. 1978. Cobalamin binding and cobalamin-dependent enzyme activity in normal and mutant human fibroblasts. *J. Clin. Invest.* 62:952-960.
- Mellman, I., H. F. Willard, P. Youngdahl-Turner, and L. E. Rosenberg. 1979. Cobalamin coenzyme synthesis in normal and mutant human fibroblasts; evidence for a processing enzyme activity deficient in cbl C cells. *J. Biol. Chem.* 254:11847-11853.
- Schneider, E. L., E. J. Stanbridge, and C. J. Epstein. 1974. Incorporation of 3H -uridine and 3H -uracil into RNA. A simple technique for the detection of mycoplasma contamination of cultured cells. *Exp. Cell Res.* 84:311-318.
- Rosenblatt, D. S., and R. W. Erbe. 1973. Reciprocal changes in the levels of functionally related folate enzymes during the culture cycle in human fibroblasts. *Biochem. Biophys. Res. Commun.* 54:1627-1633.
- Rosenblatt, D. S., B. A. Cooper, S. Lue-Shing, P. W. K. Wong, S. Berlow, K. Narisawa, and R. Baumgartner. 1979. Folate distribution in cultured human fibroblasts. Studies on 5,10- CH_2 - H_4 PteGlu reductase deficiency. *J. Clin. Invest.* 63:1019-1025.
- Shapiro, S. K., and D. J. Ehninger. 1966. Methods for the analysis and preparation of adenosylmethionine and adenosylhomocysteine. *Anal. Biochem.* 15:323-333.
- Linnell, J. C., H. M. MacKenzie, J. Wilson, and D. M. Matthews. 1969. Patterns of plasma cobalamins in control subjects and in cases of vitamin B_{12} deficiency. *J. Clin. Pathol. (Lond.)* 22:545-550.
- Tortolani, G., P. Bianchini, and V. Mantovani. 1970. Separation and determination of cobalamins on an SP-Sephadex column. *J. Chromatog.* 53:577-579.
- Jacobsen, D. W., R. Green, E. V. Quadros, and Y. D. Montejado. 1982. Rapid analysis of cobalamin coenzymes and related corrinoid analogs by high-performance liquid chromatography. *Anal. Biochem.* 120:394-403.
- Begley, J. A., and C. A. Hall. 1979. Presence of sulfitecobalamin in cell extracts. Resolution and identification by SP-Sephadex C25 Cation exchange chromatography. In *Vitamin B₁₂*. B. Zagalak and W. Friedrich, editors. W. de Gruyter, Berlin. 971-974.
- Blackburn, R., M. Kyaw, and A. J. Swallow. 1977. Reaction of Cob(I)alamin with nitrous oxide and cob(III)alamin. *J. Chem. Soc. Faraday Trans.* 73:250-255.
- Taylor, R. T. 1982. B_{12} -dependent methionine biosynthesis. In *B₁₂ Volume 2*. David Dolphin, editor, John Wiley & Sons, Inc. 307-355.
- Fujii, K., and F. M. Hunnekens. 1974. Activation of methionine synthetase by a reduced triphosphopyridine nucleotide-dependent flavoprotein system. *J. Biol. Chem.* 249:6745-6753.
- Youngdahl-Turner, P., L. E. Rosenberg, and R. H. Allen. 1978. Binding and uptake of transcobalamin II by human fibroblasts. *J. Clin. Invest.* 61:133-141.